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Note

Closely-related *Xanthomonas citri* subsp. *citri* isolates trigger distinct histological and transcriptional responses in *Citrus limon*

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ABSTRACT: Citrus canker, caused by *Xanthomonas citri* subsp. *citri* (*Xcc*), has an important economic impact on the citrus industry. Extensive information is available about the disease but, nevertheless, the study of plant-pathogen interactions could provide new information in the understanding of citrus canker disease. A new isolate has been identified, *Xcc* A^T, which has a high genetic similarity (> 90 %) to the virulent *Xcc* T strain based on genetic clustering analyses of the rep-PCR fingerprinting patterns, but it does not produce cankerous lesions in *Citrus limon*. In this study, we compared *C. limon* responses to *Xcc* A^T and to the virulent *Xcc* T strain at both histological and transcriptional levels. Histologically, leaves inoculated with *Xcc* A^T exhibited neither a typical disordering of the spongy mesophyll, nor a swelling of epidermis. A particular content (undetermined) was also found in mesophyll cells near the stomata, together with increased starch accumulation. The transcriptomic profiles were compared by cDNA-AFLP technique. A total of 121 fragments derived from transcript (TDF) were either specifically induced or repressed by the isolates, and 62 were sequenced. Analysis of global expression identified different classes of genes known to be involved in plant-pathogen interactions. This study constitutes the first approach of the specific interaction between the avirulent *Xcc* A^T isolate and *C. limon*.
Keywords: cDNA-AFLP, gene expression, canker, plant defense, microscopy

Introduction

Asiatic citrus canker, caused by the bacterial plant pathogen *Xanthomonas citri* subsp. *citri* (*Xcc*), is a widespread disease that affects most commercial citrus species and varieties. *Xcc* infects hosts through natural openings, such as stomata and lesions in plant tissue, producing pustule-like cankers on leaves, stems and fruits (Brunings and Gabriel, 2003) and defoliation, blemished fruit, premature abscission and fruit drop in severe infections, all of which results in crop loss. Because *Xcc* is a quarantine pathogen in many countries, the impact of the disease on commerce is the most serious consequence, as there are restrictions on interstate and international fruit trade from canker-affected areas are imposed (Gottwald et al., 2002; 2009).

The disease is present in the Americas, where it first appeared in Brazil, and soon spread to other important citrus producing countries, such as Argentina, one of the world's largest lemon producers. Extensive information is already available about the disease, its epidemiology, the susceptibility of host genotypes and, in particular, the *Xcc* pathogen (Gottwald, 1993; Graham et al., 2004; Van Sluys et al., 2002; Vojnov et al., 2010). However. the study of plant-pathogen interactions could provide new information in the understanding of citrus canker disease.

Chiesa et al. (2013) identified a new *Xcc* isolate with a high genetic similarity (> 90 %) to the virulent *Xcc* T strain based on genetic clustering analyses of the fingerprinting patterns generated by BOX, repetitive extragenic palindromic (REP), and enterobacterial repetitive intergenic consensus (ERIC) polymerase chain reaction (PCR) (repetitive elements-based [rep]-PCRs). Named *Xcc* A^T, this isolate does not produce cankerous lesions in a host-specific interaction with *C. limon*, and is deficient in extracellular polysaccharide (EPS) xanthan production and induces an oxidative burst and a defense response in *C. limon*, independent of supplementation with exogenous xanthan (Chiesa et al., 2013).

In this study, we compared *C. limon* responses to *Xcc* A^T and the virulent *Xcc* T strain at both histological and transcriptional levels. The close genetic relationship between these two isolates could serve to understand the mechanism underlying resistance and susceptibility.

Materials and Methods

Plant material and inoculum preparation

Six-month plants of *Citrus limon* (L.) Burm cv. Frost Eureka Nuclear were used to reproduce canker disease under controlled growth conditions. Plant leaves were inoculated with a bacterial suspension of *Xcc* A^T or virulent *Xcc* T strain with a concentration of 10^8 CFU mL^{-1}, using swab methods to mimic a natural *Xcc* infection process (Sendín et al., 2011). Inoculation with magnesium chloride ($MgCl_2$) was used as control. After inoculation, plants were covered with plastic bags and kept overnight, and then transferred to a growth chamber at 28 °C, with 70 % relative humidity and a 16 h photoperiod.

Light microscopy and cDNA-AFLP analysis

For histological analyses, samples of inoculated leaves were taken at 0, 3 and 7 days post-inoculation (dpi). Cross sections of these samples were stained with safranin, methyl blue and iodine (D`Ambrogio de Argüeso, 1986), mounted on slides in a solution of glycerin / water (50/50) and observed in a light microscope.

For transcriptional analyses, total RNA from inoculated leaves was prepared by LiCl precipitation (Sambrook et al., 1989). The cDNA-AFLP-based transcript profiling procedure was followed as prescribed by Vuylsteke et al. (2007), but with different restriction enzymes (MseI and *Eco*RI). Selective amplifications using the primers 5′-GACTGCGTACCAATTCANN-3′ (*Eco*RI, where NN represents –GG, CT, GC, CC, AG, CA, AC and CG) and 5′-GATGAGTCCTGAGTAACMM-3′ (MseI, where MM represents TT, AT, AC, AA TC, TA, AG and TG) were performed with a total of 64 selective primer combinations. Amplification products were separated on 5 % polyacrylamide gels, reamplified and sequenced. Sequences obtained were compared with those shown by the BLASTn and BLASTx (NCBI, http://www.ncbi.nlm.nih.gov/BLAST), PHYTOZOME (http://www.phytozome.net), "ORANGE (*Citrus sinensis*) genome annotation" (http://citrus.hzau.edu.cn) and HARVEST (http://cgf.ucdavis.edu) searching engines. The expression level of eight TDFs randomly selected from Table 1 was quantified by quantitative real time retrotranscription-PCR (qRT-PCR) to validate the pattern of TDFs obtained with cDNA-AFLP. qRT-PCR analyses were performed in a 25 µL volume containing 1X master mix (iQ SYBR Green), 0.4 uM of each primer, and 4 µL of 10-fold cDNA dilutions. The relative expression of RNA transcripts was quantified with the threshold cycle value (Ct) obtained from each sample by using the 2-ΔΔCt method (Livak and Schmittgen, 2001), with a plant cytochrome oxidase (COX) gene for reference gene expression (Li et al., 2005).

Results and Discussion

Symptomatology and histology reveal differences in the interaction between *C. limon* and the closely-related *Xcc* A^T and *Xcc* T

Differential symptomatology was observed in young *C. limon* leaves treated with avirulent (*Xcc* A^T) or virulent (*Xcc* T) isolates. Seven days post-inoculation (7 dpi), *Xcc* T produced the typical circular water soaked lesions, in contrast with leaves inoculated with *Xcc* A^T, where circular dark spots were observed (Figure 1).

The differential symptomatology observed in *C. limon* after inoculation with *Xcc* T or *Xcc* A^T was histologically analyzed by light microscopy. In leaf samples inoculated with *Xcc* T, it was observed that cells around the stomata were disorderly, intercellular space in the spongy mesophyll region had diminished, and leaf epidermal cells had swollen by 3 dpi (Figure 2A, B). By 7 dpi typical hyperplasia and hypertrophy of mesophyll cells were observed, with a consequent rupture of abaxial epidermis and bacteria oozing at the site of infection (Figure 2D, E). In contrast, in leaf samples inoculated with *Xcc* A^T (Figure 2G, H, J), as well as in control leaves (Figure 2M, N, P, Q), the spongy mesophyll did not show disordering and epidermal cells had not swollen either by 3 dpi nor 7 dpi. However, the mesophyll cells near the stomata showed a particular content by 7 dpi in leaves inoculated with *Xcc* A^T (not determined) (Figure 2K). The starch accumulation was higher in guardian cells of stomata in leaves inoculated with *Xcc* A^T, than in leaf tissue inoculated with *Xcc* T (Figure 2C, F, I, L). The highest amount of starch was observed at 3 dpi, but it dropped at 7 dpi (Figure 2I, L). In control leaves, cumuli of starch were not observed (Figure 2O, R). This reaction has been cited as a general plant response to different pathogen infections (Bertani et al., 2014; Pardo et al., 2012). Some evidence suggests that the wild *Xcc* strain presents in its

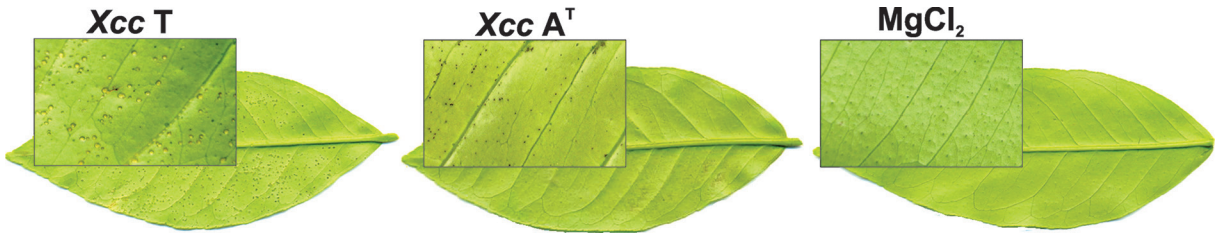

Figure 1 – Differential symptomatology induced in *Citrus limon* by *Xcc* T and *Xcc* A^T. Foliar symptoms were observed at 7 days post-inoculation (dpi) in leaves inoculated with either *Xanthomonas citri* subsp. *citri* isolates, *Xcc* T (left) or *Xcc* A^T (middle), and in mock inoculation with $MgCl_2$ (right).

Table 1 – Similarities of sequences of *Citrus limon* differentially expressed in response to either *Xanthomonas citri* subsp. *citri* isolates (*Xcc* T or *Xcc* A^T) infection.

	Length	Accession number of Highest similarity		E value
TDFs induced by Xcc A^T				
Cit 11	361	AAG49361.1	Aminocyclopropanecarboxylate oxidase	6.29E-02
Cit 32	200	Orange1.1 g 022529 m.g	Chlorophyll a/b binding protein	2.90E-14
Cit 34	199	Cs4g03600	Plant recepetor-like Serine treonine kinase	5.8E-02
Cit 35	162	Orange1.1 g 022529 m.g	Mis 12 protein	3.90E-05
Cit 40	154	Orange1.1 g 024610 m.g	ATP10 Protein	6.55E-02
Cit 42	106	EF377537.1	Messenger RNA from leaves of *Poncirus trifoliata*	7.00E-07
Cit 76	102	EY734971.1	Sweet orange fruit, development stadium, *Citrus sinensis* cDNA	4.00E-04
Cit 77	103	EU442614	*Citrus reticulata* amplified fragment length polymorphism marker	1.8E-02
Cit 78	107	Orange1.1 g 011573 m.g	ATPase family associated with various celular activities (AAA)	4.16E-02
Cit 105	135	Orange1.1 t 03117	Putative beta-galactosidase 10	0.54
Cit 108	149	Orange1.1 g 019667 m.g	PRP38, splicing factor	2.90E-06
TDFs induced by Xcc T				
Cit 3	206	ABL67650.1	Miraculin like protein	0.58
Cit 4	178	EY701345.1	Sweet orange fruit, development stadium, *Citrus sinensis* DNA copy	0.36
Cit 6	126	Orange1.1 g 008135 m.g	HEC1 protein	1.48E-02
Cit 7	200	EY823070.1	*Poncirus trifoliata* leaf, infected with Citrus tristeza virus Citrus trifoliata cDNA, mRNA sequence	3.0E-03
Cit 26	212	Orange1.1g025671 m.g	Chlorophyll a/b binding protein	7.20E-11
Cit 27	204	Orange1.1g015983 m	Leucine rich-repeat containing protein	6.30E-138
Cit 28	199	EF377537.1	*Poncirus trifoliata* leaves 1 year old plants Citrus trifoliata cDNA clone S134 similar to phospho-enolpyruvate	6.00E-18
Cit 29	80	GO787079.1	*Citrus unshiu* cDNA clone CuD09 similar to lipoxygenase, mRNA sequence	0.13
Cit 41	126	Orange1.1 g 038028 m.g	Ciclin H	1.10E-10
Cit 43	60	Cs2g10510	MOR1 protein	0.2
Cit 44	197	Cs9g08940	ARP8 protein	0.053
Cit 55	55	Cs7g08120	DEAD-box ATP-dependent RNA helicase	0.18
Cit 57	199	HQ884142.1	*Linum* sp. microsatelite sequence	1.00E-06
Cit 63	206	Orange1.1 g 044905 m.g	Nitrogen metabolic regulation protein- related	2.00E-20
Cit 64	207	Cs9g08120	Putative CVP2 like I inositol-1,4,5-trifosfate 5-fostfatase	1.00E-27
Cit 67	107	Orange1.1 g 017810 m.g	Putative serine esterase	2.60E-17
Cit 72	216	Scaffold00052	Uncharacterized protein	5.10E-56
Cit 74	202	Orange1.1 g 017810 m.g	Ribosomal L7/L12 protein	2.30E-09
Cit 85	566	EF377537.1	Fosfoenol piruvate carboxilase	1.00E-12
Cit 86	200	Orange1.1 g 017810 m.g	GTPase protein	8.95E-02
Cit 89	200	Cs2g030400	Photosystem II related protein	7.00E-26
Cit 92	402	CX636796.1	mRNA sequence from *Poncirus trifoliata*	3.00E-134
Cit 94	378	Orange1.1 g 037191 m.g	Uncharacterized protein	1.10E-73
Cit 102	406	Cs6g08490	MYB transcrition factor	1.00E-95
Cit 104	136	Cs6g03890	1-O-Acylglucosa antocianin O-Acyltransferase	1.00E-04
TDFs repressed by Xcc A^T				
Cit 1	416	orange1.1 g 018057 m.g	Transcription initiation factor TFIID subunit 8	3.60E-67
Cit 5	174	EY732374.1	mRNA sequence from *Citrus sinensis*	0.1
Cit 8	103	ABX46354.1	Resistance protein like nucleotide binding site-Leucine-rich repeat	3.10E-02
Cit 9	77	Orange1.1 g 000128 m.g	Leucine rich- repeat protein	1.50E-05
Cit 16	87	DN799236.1	mRNA sequence from *Citrus reticulata* × *Citrus temple*	1.00E-17
Cit 25	200	CD585758.1	*Poncirus trifoliata* leaf, infected with CTV Citrus trifoliata cDNA, mRNA sequence	1.00E-26
Cit 62	197	Orange1.1 g 0479 m.g	Serine/treonine Protein Kinase	1.00E-24
Cit 65	160	XM_006470462.1	*Citrus sinensis* protein AATF-like	0.003
Cit 70	70	EY675277.1	*Poncirus trifoliata* leaf, infected with CTV *Citrus trifoliata* cDNA, mRNA sequence	0.36
Cit 90	197	Orange1.1 g 022614 m.g	SNARE protein	3.10E-20
Cit 103	128	CAP40294.1	Putative beta galactosidase	0.18
TDFs repressed by Xcc T				
Cit 12	52	AAK83559.1	pY65, Putative resistance gene	6.60E-01
Cit 33	176	ADE05307.1	Actin protein	0.41
Cit 97	209	Scaffold00138	Uncharacterized protein	1.70E-55

*Those TDFs (fragments derived from transcripts) that not have similarities with known sequences were excluded from the table.

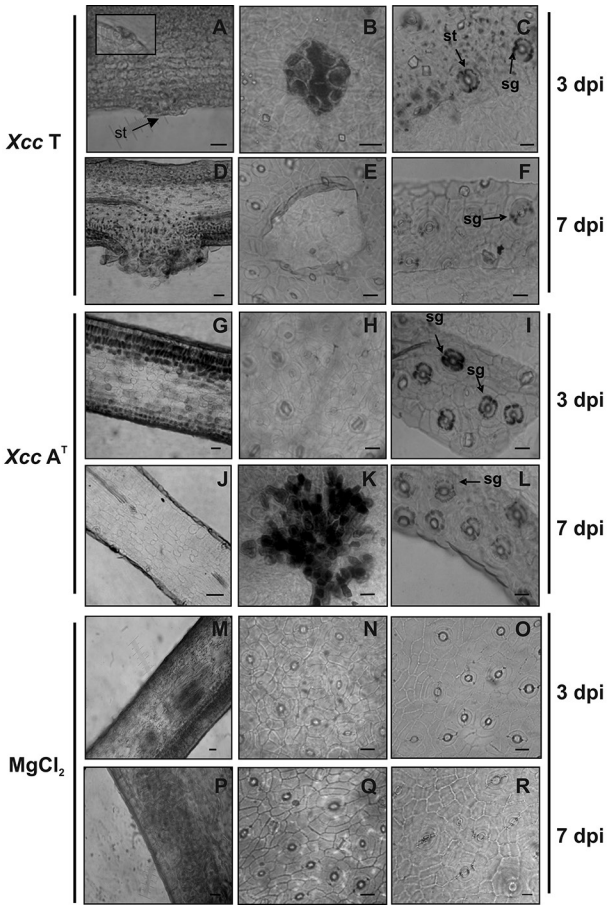

Figure 2 – Histopathologic analysis of *Citrus limon* leaves inoculated with virulent (*Xcc* T) or avirulent (*Xcc* A^T) *Xanthomonas citri* subsp. *citri* isolates on the 3 and 7 day post-inoculations (dpi). Cross-sections of leaves inoculated with *Xcc* T (A, D) and *Xcc* A^T (G, J), and control leaves (M, P); a detailed close-up of stomata is included as insert in A. Paradermal views of leaves inoculated with *Xcc* T (B, E), *Xcc* A^T (H, K), or control leaves (N, Q). Histochemical Lugol test in leaves inoculated with *Xcc* T (C, F), *Xcc* A^T (I, L) or control leaves (O, R). Arrows indicate stomata (st) or starch accumulation (sg); scale bars: 15 µm.

genome a gene coding for a protein similar to natriuretic plant proteins, which are involved in the regulation of cell growth and homeostasis (Gottig et al., 2008). This protein was shown to alter host physiological responses by means of starch granule degradation in the stomatal guard cells of the plant, causing stomatal openings thereof (Gottig et al., 2008). It is possible that this protein is defective or absent in *Xcc* A^T, leading to starch accumulation in stoma cells which then hamper the entry of the pathogen.

The avirulent *Xcc* A^T elicits a distinct gene expression pattern in the interaction with *C. limon*

To find out what genes are involved in the interaction between *C. limon* and the new *Xcc* A^T isolate, a transcript profile was attained through cDNA amplified fragment length polymorphism (cDNA-AFLP) technique, as a first approach to elucidate certain of the mechanisms involved in this specifically induced interaction. This study was carried out 48 h post-inoculation (hpi), comparing the gene profile induced by the avirulent *Xcc* A^T isolate with those produced by the virulent strain (*Xcc* T) or mock inoculation with $MgCl_2$. Out of 752 fragments derived from transcripts (TDFs) observed in silver-stained cDNA-AFLP gels, 121 TDFs were specifically induced or repressed by virulent (*Xcc* T) and avirulent (*Xcc* A^T) isolates. Most of the up-regulated TDFs were observed when the plant was challenged with the virulent isolate *Xcc* T (47 %); the reverse was true for down-regulated TDFs, as their lowest number was recorded in this case (6 %) (Table 1). By contrast, in the interaction with the *Xcc* A^T isolate, the numbers of up-regulated (26 %) and down-regulated (21 %) TDFs were similar (Table 1). A total of 62 differential TDFs sequences were successfully obtained. Table 1 shows the similarities of TDFs differentially expressed in response to either *Xcc* T or *Xcc* A^T infection. The expression level of eight TDFs randomly selected from Table 1 was validated by qRT-PCR (Figure 3). As much as 18 % of the TDFs did not show any significant similarity to known proteins. The next most abundant group was composed of TDFs with significant similarity to resistance genes (15 %) and genes involved in plant metabolism (15 %). The other TDFs were identified as being similar to genes involved in transcription and translation (13 %), energy (10 %), pathway signaling (5 %), structural proteins (5 %) and transport mechanisms (2 %). Other TDF groups were linked with citrus EST (11 %) or uncharacterized proteins (8 %) in the database.

Some TDFs differentially expressed in the interactions are involved in the same mechanisms; for example, genes encoding proteins of the light-harvesting complex of photosystem II (PSII) (Table 1, Cit 26, Cit 32 and Cit 89). It has been shown that the PSII system plays an important role in preventing the accumulation of reactive oxygen species (ROS) (Asada, 1999), and it also seems to participate in both compatible and incompatible plant pathogen interactions (Baldo et al., 2010).

In the interaction with the virulent isolate, up-regulated genes involved in cell cycle, growth and differentiation (and related with hyperplasia and hypertrophy) were identified, as has been previously described (Cernadas et al., 2008).

Interestingly, one of the TDFs induced by *Xcc* T showed sequence similarity to an MYB transcription factor (Cit 102). Baldo et al. (2010) also found a TDF homologue to MYB 11 in *Malus domestica*, which was overexpressed in susceptible cultivars after inoculation with *Erwinia amylovora*. In the same way, two MYB genes in *Arabidopsis thaliana* were specifically induced by Type 3 effector proteins, and they negatively regulated phenylpropanoid metabolism (Preston et al., 2004). They are widely distributed in plants and have been reported to be involved in the ABA-response and to have interactions

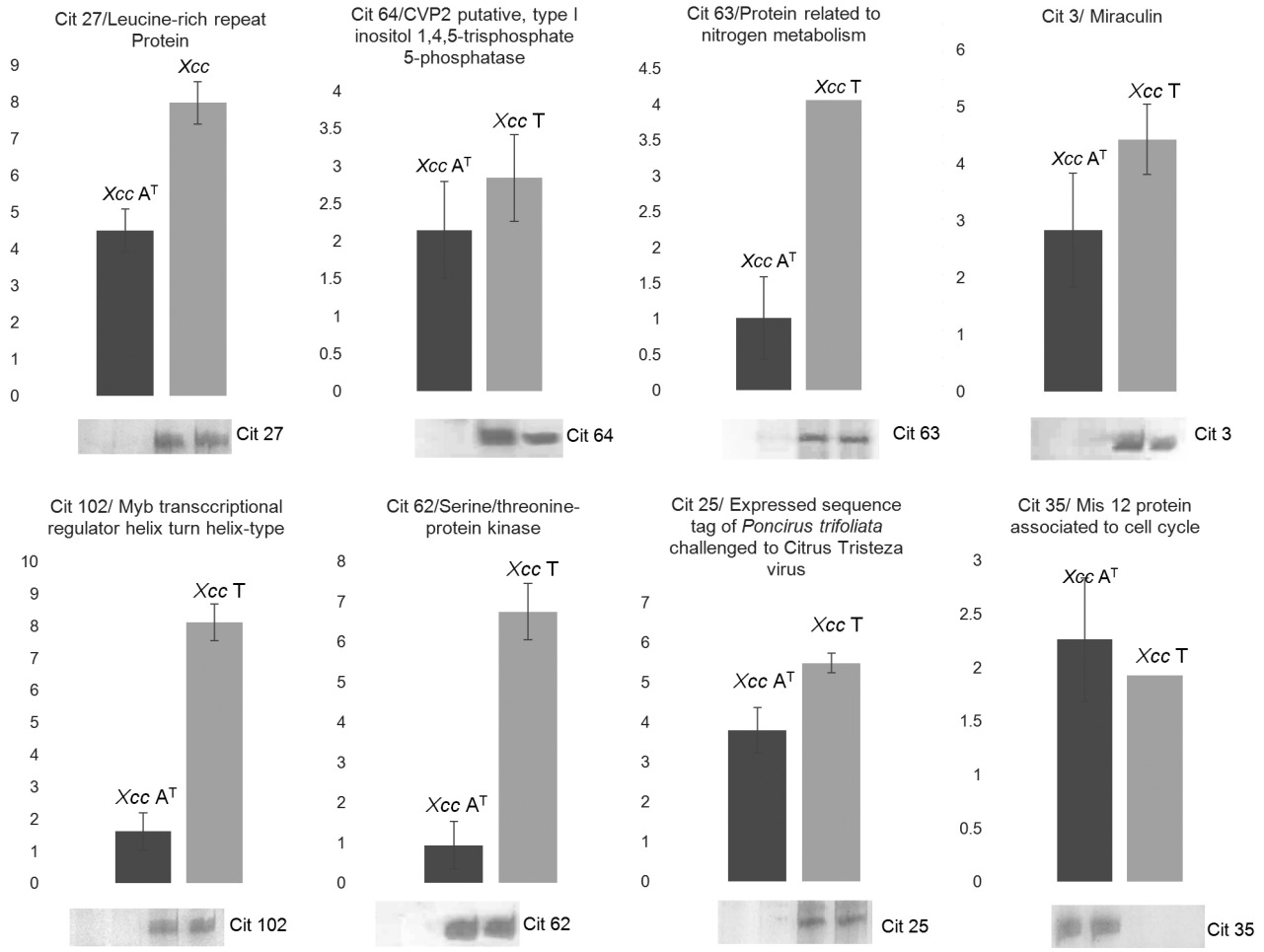

Figure 3 – Quantitative real time retrotranscription- PCR (RT-PCR) analysis of randomly selected transcript derived fragments (TDF) expressed diferentially during *Xcc/ Citrus limon* interaction. Relative expression of 8 randomly selected TDF (Cit 27, 64, 63, 3, 102, 62, 25 and 35) was quantified through quantitative real time RT-PCR using the $2^{-\Delta\Delta Ct}$ method to validate the expression level observed in cDNA-AFLP analysis. All data were normalized using a plant cytochrome oxidase (COX) gene. The mean expression value was calculated for every TDF with three replications. The corresponding signal in a cDNA-AFLP analysis is included for each target.

with other transcription factors (Ambawat et al., 2013). In this interaction, one TDF similar to a lipoxygenase (Cit 29) was also induced. Lipoxygenase (LOX) has been identified as generating highly cytotoxic compounds like unsaturated aldehydes and lipid hydroperoxides, which interact with transition metals to produce corrosive radicals (Brash, 1999). These compounds could produce damage in the membrane by an alternative way to the oxidative burst produced during hypersensitivity reactions (HR) of an incompatible interaction (Adam et al., 1989; Porter et al., 1995; Mittler et al., 1996). However, the LOX mechanism has been proven to be associated with plant-pathogen interaction, and has been observed in both compatible and incompatible interactions (Jalloul et al., 2002). In concordance with Chiesa et al. (2013), we could say that it is possible that *Xcc* T inactivates ROS mechanism; nonetheless, the plant can activate another defense pathway, such as the LOX mechanism.

As expected, many of the TDFs expressed differentially during the interaction between *C. limon* and *Xcc* A^T were found to be related in some way to resistance and plant defense response. *Xcc* A^T induced a TDF similar to plant receptor-like serine threonine kinase (RLSTK) (Cit 34). This receptor recognizes molecules of pathogens and activates signals triggering plant defense responses; such is the case of rice bacterial blight resistance gene product XA21 (Song et al., 1995) and FLS2, a flagellin perception gene in *A. thaliana* (Gómez-Gómez and Boller, 2000). Another of the TDFs induced by *Xcc* A^T was Cit 11, which shows similarity to the enzyme aminocyclopropanecarboxylate oxidase (ACC oxidase), which in turn catalyzes the cleavage of 1-aminocyclopropane-1-carboxylic acid (ACC) into ethylene. It is widely known that ethylene, together with other compounds such as salicylic acid, phytoalexins or reactive oxygen species (ROS), forms part of the complex plant defense mechanism (Joshi and Nayak,

2011). ACC oxidase induction could indicate that ethylene increases with the interaction between *C. limon* and this avirulent isolate. The activation of this gene in citrus plants was previously described in sweet orange Pera inoculated with *X. axonopodis* pv. *aurantifolii* (*Xaa*) pathotype C, which does not produce canker in this genotype. *Xaa* induces ethylene related genes like ACC oxidases, as well as ethylene receptors, ethylene-induced esterase and ethylene response factors 48 hpi, and these responses are associated to disease resistance (Cernadas et al., 2008).

Interestingly, *Xcc* A^T induced the expression of a TDF similar to a PRP38 splicing factor (Cit 108). Recently, new findings have provided insights into the roles of alternative splicing in the regulation of expression of plant defense genes (Marone et al., 2013). This factor has been generally associated with abiotic stresses such as cold and salinity (Forment et al., 2005; Iida et al., 2004); however, this is the first report of this gene in association with a biotic factor.

On the other hand, in the interaction between *C. limon* and *Xcc* A^T a TDF similar to a gene coding for SNARE proteins (Cit 90) was suppressed. One of the primary roles of SNARE proteins is to mediate fusion between cellular transport vesicles and the cellular plasma membrane for exocytosis. A study using inhibitors of vesicle trafficking has shown that this inhibition delays pustule development induced by *Xcc* in sweet orange Pera leaves (Cernadas et al., 2008), so the suppression of this gene would be consistent with the lack of symptoms in *Xcc* A^T inoculated leaves.

In conclusion, unlike the virulent type, the avirulent isolate *Xcc* A^T did not prevent the development of physical barriers, such as starch accumulation or cell wall thickening, both events associated with plant defense mechanisms. Differences in gene expression patterns of infected plants, together with the close genetic relationship between these two isolates, could serve as the basis for developing different hypotheses related to the resistance or susceptibility mechanisms responsible for the different infection patterns that these two isolates exhibit. However, a high-throughput sequencing approach will be needed to get a more complete picture of differences in the plant molecular response to both *Xcc* isolates.

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